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A human neutralizing antibody against a conformational epitope shared by oligomeric SARS S1 protein

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An antibody phage-display library was constructed from the B cells of convalescent severe acute respiratory syndrome (SARS) patients and screened using inactivated SARS coronavirus (CoV) virions as antigens. More than 80 positive clones were isolated from the library and one of them, scFv H12, was extensively characterized. scFv H12 bound to SARS-CoV with high affinity (equilibrium dissociation constant, K_d=73.5 nM), and neutralized SARS virions *in vitro*. The facts that scFv H12 bound to the SARS-S1 protein under non-reducing conditions and that it did not bind to monomeric S1 protein under reducing conditions strongly suggest that scFv H12 recognizes a conformational epitope shared by oligomeric S1 proteins. This study should aid in the manufacture of neutralizing antibody, provide a better understanding the immunological characteristics of SARS protein and facilitate the design of a SARS vaccine.

Introduction

Severe acute respiratory syndrome (SARS) is a recently emerged disease caused by SARS-associated coronavirus (SARS-CoV) and representing a serious threat to human health [1–3]. The SARS-CoV is highly contagious and the clinical course of SARS is severe with high mortality. During the worldwide outbreak in 2003, there was no effective agent available for SARS prophylaxis and therapy [4,5]. Although passive serotherapy using polyclonal immune sera from convalescent SARS patients has been shown to be an efficient treatment for SARS and potentially provides immediate protection against infection [6–8], human sera stocks are very limited and are potentially contaminated with infectious agents. Therefore, an alternative viable strategy for SARS prophylaxis and treatment is needed to produce human neutralizing antibodies against SARS by antibody engineering technology.

It has been reported that the N-terminus of the spike (S) protein of SARS-CoV can form a dimer and the ectodomain can form a trimer. This oligomeric state of the S protein is important for its binding to the cell surface receptor to initiate entry of virus into host cells [9,10]. Our previous study [11] showed an image of SARS-CoV in which the surface bears flower-shaped projections containing a trimer of the S proteins. Besides binding to its receptor angiotension-converting enzyme 2 (ACE2) [12,13], the S protein has been identified as a dominant antigenic determinant eliciting neutralizing antibodies during infection with SARS-CoV [14,15]. Several antibodies with neutralizing activities to block SARS-CoV infection, which have been selected by using the S protein as antigen from naive [16], semisynthetic antibody libraries [17] and memory repertoire [18], have shown that the epitope is not conformation-dependent. This is not consistent with some results from SARS convalescent serum [19,20]. A more directed verification other than SARS convalescent serum is needed.

In the present study, we generated a SARS-CoV immunized antibody library and isolated a single-chain Fv (scFv) with high specificity and affinity by screening the library using whole SARS-CoV virions as antigen. Our results indicated that the selected scFv H12 has potential neutralizing activity by recognizing a conformational epitope within oligomeric S1 proteins. This study should aid in the manufacture of neutralizing antibody, which should mimic natural antibody in immune sera in recognizing a naturally immunogenic determinant. This study should also provde a better understanding of the immunological characteristics of SARS protein and facilitate the design of SARS vaccine.

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Materials and methods

Cells, plasmids and antibodies

Vero E6 cells were supplied by the Institute of Microbiology and Epidemiology (Beijing, China). pET28a (+) vector was purchased from Novagen (Darmstadt, Germany). Phagemid pDNA5 [21] was provided by Andrew Bradbury from Los Alamos National Laboratory (Los Alamos, NM, USA). pCDM8/S1-Fc vector [12] was provided by Hyeryun Choe of Harvard Medical School (Boston, MA, USA). The antibodies used in this study included horseradish peroxidase (HRP)-conjugated anti-M13 from Amersham Pharmacia (Amersham, UK), HRP-conjugated anti-human Fc IgG from Sigma (St Louis, MO, USA), HRP-conjugated anti-mouse IgG from Pierce (Rockford, IL, USA) and anti His-Tag from Novagen.

Virion preparation

SARS-CoV BJ01 were inactivated in a biosafety level 3 laboratory and purified by differential centrifugation as previously described [11].

Construction of scFv library

Total RNA was prepared from peripheral blood lymphocytes of four convalescent SARS patients, and followed by cDNA synthesis. The heavy-chain variable region (V_H) and light-chain variable region genes (V_L) were amplified using the primers listed in Table 1, and then cloned into phagemid pDAN5 [21]. The recombinant pDAN5 was transformed into *Escherichia coli* XL1-Blue by electroporation. The transformants were plated on dishes with 2×YT, containing 1% glucose, 100 μg/ml ampicillin and 10 μg/ml tetracycline. All colonies were collected by scraping, rescued by helper phage M13KO7 [22], and finally stored as a primary scFv library.

Diversity assessment of the scFv library

A number of individual colonies from the primary library were randomly picked and their phagemids with scFv were prepared as previously described [23]. After amplification using PCR, the individual scFv gene was digested by *Bst*NI, and the fingerprinting of each colony was analysed by agarose gels.

Selection of phage scFv to SARS-CoV

The immunotubes were coated with purified SARS-CoV virions in 0.05 M Na_2CO_3 pH 9.6, blocked with 3% bovine serum albumin (BSA) in PBS for 2 h, and incubated with 10^{12} phage scFv in 1 ml PBS containing 1% BSA and 0.1% Triton X-100 at room temperature for 3 h. After intensive washes with PBST (0.1% Tween-20 in PBS), bound phage antibodies were eluted

Table 1. A set of human antibody primers for amplification of V_H and V_L genes was designed based on previous publications [22–23,29–31]

Primer	Sequence
Human V_H Back primers with XhoI	
V_{H1} Back XhoI	TTATCCTCGAGCGGTACCSAGGTSCAGCTGGTRCAGTCTGG
V_{H2} Back XhoI	TTATCCTCGAGCGGTACCCAGRTCACCTTGAAGGAGTCTG
V_{H3} Back XhoI	TTATCCTCGAGCGGTACCSAGGTGCAGCTGKTGSAG
V_{H4} Back XhoI	TTATCCTCGAGCGGTACCCAGSTRCAGCTRCAGSAGTS
Human V_H for primers with Nhe I cite	
J_H For Nhe I	GATTGGTTTGCCGCTAGCTGARGAGACRGTGACCRKKGT
Human $V_κ$ Back primers with BssH II	
$V_κ$1 Back BssH II	AGCAAGCGGCGCGCATGCCGMCATCCRGDTGACCCAGTCTCC
$V_κ$2 Back BssH II	AGCAAGCGGCGCGCATGCCGATATTGTGMTGACBCAGWCTCC
$V_κ$3 Back BssH II	AGCAAGCGGCGCGCATGCCGAAATTGTGHTGACDCAGTCTCC
$V_κ$4 Back BssH II	AGCAAGCGGCGCGCATGCCGAAACKACACTCACGCAGTCTC
Human $V_κ$ for primers with Sal I	
$J_κ$1 for Sal I	GAAGTTATGGTCGACCCTCCGGAACGTTTGATHTCCASYTTGGTCC
$J_κ$2 for Sal I	GAAGTTATGGTCGACCCTCCGGAACGTTTAATCTCCAGTCGTGTCC
Human $V_λ$ Back primers with BssH II	
$V_λ$1Back BssH II	AGCAAGCGGCGCGCATGCCCAGYCWGYBYTGAYKCAGCC
$V_λ$2Back BssH II	AGCAAGCGGCGCGCATGCCTCCTMTGWGCTGABDCAGS
$V_λ$3Back BssH II	AGCAAGCGGCGCGCATGCCAATTTTATGCTGACTCAGCCCC
Human $V_λ$ for primers with Sal I	
$J_λ$ for Sal I	GAAGTTATGGTCGACCCTCCGGAACCTAGGACGGTSASCTTGGTCCC

Italic indicates the flank sequence of primers, underline indicates the restriction enzyme site, and bold indicates the primer sequence for amplifying the genes of human V_H and V_L.

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with 0.1 M glycine/HCl pH 2.2 and immediately neutralized with 1.0 M Tris-Cl pH 8.0. Eluted phage scFv were subjected to the next round of infection, rescue and selection. After five rounds of panning, the strong binders to SARS-CoV were selected by ELISA.

ELISA

96-well microtitre plates (Nunc, Rochester, NY, USA) were coated overnight at 4°C with inactivated SARS-CoV particles in 0.05 M Na_2CO_3 pH 9.6, blocked with 3% BSA in PBS, and incubated with an individual phage scFv in PBS containing 1% BSA. After five washes with PBST, the bound antibodies were detected by HRP-conjugated anti-M13 antibody followed by incubation with ortho-phenylene-diamine (OPD) as substrate. The colour reaction was measured at 490 nm in a BioRad ELISA reader (Hercules, CA, USA).

Expression and purification of scFv

In order to obtain a large amount of scFv protein, the genes of selected phage scFv were cloned into pET28a (+) vector, and scFv protein was expressed with 6xHis tag in *E. coli* BL21 (DE3). The scFv in inclusion bodies was denatured with 8 M urea, and then purified and refolded during gel filtration on a column with sephacryl S200HR (10×1100 mm) as previously described [24]. Briefly, the column was loaded with sample in 8 M urea denature buffer and then run with the refolding buffer (50mM Tris-Cl, pH8.3, 0.15M NaCl, 0.4M L-arginine, 2mM EDTA, 0.1M urea, 0.4mM GST, 1mM GSSG) at a flow rate 0.2ml/min. The peaks were collected and then dialysed against the buffer containing 50mM Tris-Cl, pH8.3, 0.15M NaCl, 2mM EDTA, and finally with PBS buffer.

SDS-PAGE and western blot

The protein samples were separated by electrophoresis in SDS-PAGE and then transferred onto membrane. The membranes were blocked with 5% non-fat milk in PBS, and then incubated with a mouse anti His-Tag antibody followed by an HRP-conjugated anti-mouse IgG. The specific protein bands were visualized by autoradiography on Kodak X-ray film.

Competitive inhibition assay

Sera from convalescent SARS patients was 1,000 times diluted in PBS, and then incubated 1 h at 37°C with a serial twofold dilution of the purified scFv H12, starting at a final concentration of 17 μg/ml. The above-mentioned mixture of sera with diluted scFv H12 was subjected to 96-well plate coated with SARS-CoV particles, and incubated for another 1h at 37°C. HRP-conjugated anti-human Fc was added as secondary antibody. The following procedures were the same as described in ELISA.

SARS S1 protein preparation

The pCDM8-S1-Fc vector encoding a fusion gene of S1 protein (residues 12–672) and a human Fc of IgG_1 was transfected into 293T cells. After 48 h culture, the S1-Fc protein was produced by transfected 293T cells into culture supernatant and subjected to SDS-PAGE and Western blot for analysis.

Antibody affinity assay

The binding kinetics of scFv H12 to SARS-CoV was analysed using a variable angle spectroscopic ellipsometer. Berifly, SARS-CoV virions were covalently immobilized to a protein chip and scFv H12 solution was added on the chip surface. Binding kinetic parameters were evaluated with antibody real-time binding curve [25–27].

Preparation of HIV/SARS-S pseudovirus

The HIV/SARS-S pseudovirus was prepared as described [28]. Briefly, 293T cells were transfected with pMT 21-S, a plasmid expressing SARS-CoV S protein, pCMVR 8.2, a plasmid expressing HIV viral proteins, and pHR´-luc, a retroviral vector carrying firefly luciferase coding sequence. 48 h post infection, HIV/SARS-S pseudovirus was collected from the supernatant.

In vitro neutralization assay

HIV/SARS-S pseudovirus system was used as an infection model to evaluate the neutralizing activity of scFv H12. The HIV/SARS-S pseudovirus was pre-incubated with a serial twofold dilution of purified scFv H12, starting at a final concentration of 17 μg/ml, and used to infect Vero E6 cells. After infection for 3 h, the culture medium was replaced with fresh DMEM containing 10% FBS and cultured for another 48 h. The infected Vero E6 cells were lysed and assayed using a Luminometer DL Ready model TD2020 (Tartu, Estonia) for measuring luciferase activity in the infected cells.

Results

SARS-CoV immunized human scFv library

In order to create a large diversity of scFv library with a high affinity for SARS-CoV, we used pDNA5 as a phage-display vector and four SARS patient's lymphocytes as the repertoire of antibody. All the sera from the patients showed high titre binding to SARS-CoV. Using PCR, four groups of V_H and seven groups of V_L (four for V_κ and three for V_λ) were amplified (Figure 1A) with a set of human antibody primers (Table 1), which was optimized based on previous investigations [22–23,29–31]. The amplified V_L and V_H were each inserted into pDNA5 vector and then transformed into *E. coli* by electroporation to make a primary scFv library.

To assess the diversity of the scFv library, 13 colonies were randomly selected and their scFv genes were digested with *Bst*NI since a number of *Bst*NI sites randomly exist in the variable region of antibody. Figure 1B shows that all the 13 clones contain a similar size of scFv, around 750 bp. However, after *Bst*NI digestion, each scFv showed a unique finger-printing pattern (Figure 1C), indicating that an individual clone in the primary library is different from others. This library was calculated to have a diversity of 1.85×10^6 members.

Selection of phage antibody binding to SARS-CoV

Bio-panning was performed under stringent conditions to enrich phage scFv for SARS-CoV. The phage scFv at 10^{12} pfu (input) were subjected to immunotubes coated with SARS-CoV virions. After 3 h incubation, the immunotubes were intensively washed to remove non-specific binders, and the bound phages (output) were measured after each panning. The ratio of output/input was gradually increased after the third and fourth round, and it was dramatically increased after the fifth round. However, the phage scFv to immobilized control BSA did not show any significant changes (Figure 1D), suggesting that the phage scFv to SARS-CoV are specifically enriched after five rounds of panning.

From the fifth panning, 96 phage clones were randomly selected and further assayed by ELISA to evaluate their binding activity to SARS-CoV. We selected 86 clones that specifically bound to SARS-CoV. Among them, H12 clone was discovered to be the consistently highest binder to SARS-CoV. The H12 was identified as V_{H1} family and $V_{\kappa1}$ family based on its deduced amino acid sequence (Figure 2).

Expression and purification of H12 antibody

The scFv H12 was expressed in inclusion body of *E. coli*, purified and refolded at the same time by gel filtration through a column with sephacryl S200HR. The purified scFv H12 was analysed as a single band about 29 kDa on SDS-PAGE (Figure 3A), and detected by anti His-Tag antibody in Western blotting analysis (Figure 3B).

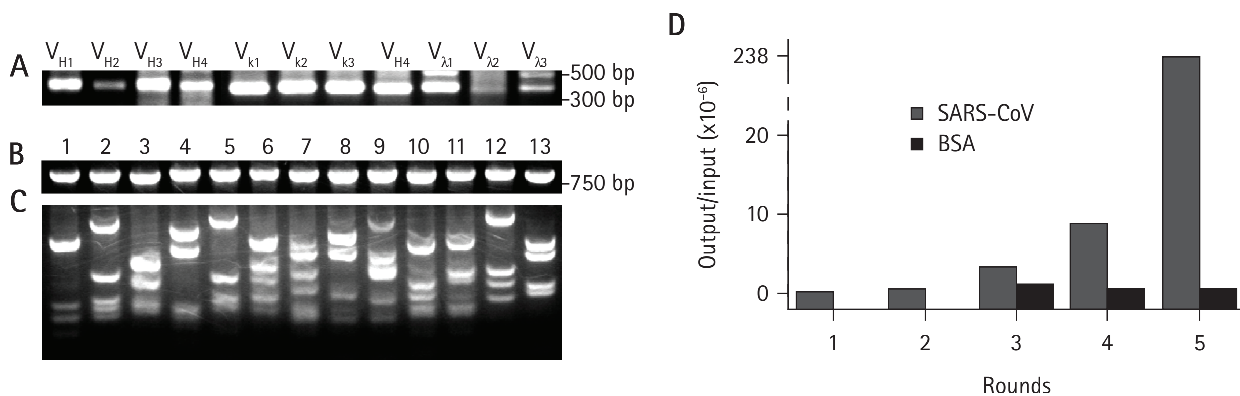
Specific binding of H12 to SARS-CoV virions

To test whether the soluble scFv H12 maintains the same binding activity to SARS-CoV as the H12 phage antibody did, we performed a competition assay using the purified scFv H12. The scFv H12 significantly abrogated the binding of SARS immune sera to SARS-CoV, and this inhibition appeared in a dose-dependent manner. In contrast, the control scFv D4 did not display any inhibitory activity (Figure 4A). The binding kinetics of scFv H12 to SARS-CoV was analysed using a variable angle spectroscopic ellipsometer. The equilibrium dissociation constant of scFv H12 was evaluated as K_d=73.5 nM with antibody real-time binding curve (Table 2).

H12 recognizes a conformational epitope on S1 protein

To map the epitope of scFv H12 on SARS-CoV, we used S1-Fc protein as antigen to test scFv H12 binding by Western blot. The S1-Fc was a fusion protein containing the S1 domain (residues 12–672) and human Fc of IgG1. As shown in Figure 3, scFv H12 specifically recognized oligomeric S1-Fc with a molecular weight around 260 kDa under non-reducing conditions, and this binding disappeared when the oligomeric S1-Fc was treated with (DTT) (Figure 4B).

Figure 1. Construction of SARS immunized scFv library



(A) Four groups of V_H and seven groups of V_L (four for V_κ and three for V_λ) were amplified from the mRNA, which was prepared from peripheral blood lymphocytes of four convalescent SARS patients. **(B)** A total of 13 scFv clones were randomly selected from the primary library and their genes were amplified by PCR. **(C)** A diversity of the primary antibody library is represented by the fingerprints of the 13 scFv genes digested with *Bst*NI that randomly exists in the variable region of antibody. **(D)** The number of the phage scFv to SARS-CoV (but not to BSA) was increasing after five rounds of panning.

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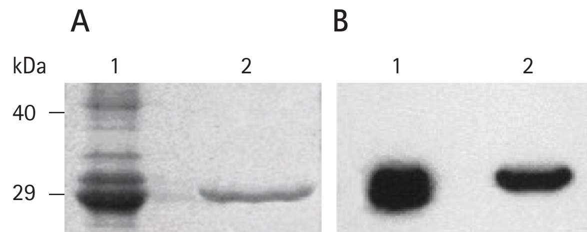
Figure 2. Amino acid sequence of scFv H12 deduced from DNA sequence

		FWR1	CDR1	FWR2	CDR2
H12V_L:	1	DIRVTQSPPSLSASIGDRVTITC-----------	RASQNIRNSLN--	WYQQKPGKAPKLLIY--	AAYSLQS------------
H12V_H:	1	EVQLVQSGAEVKKPGSSVKVSCKASGGTFS--	SHAVN---------	WVRQVPGQLEWMG---	RVIPILGTVKNAQKLQG

		FWR3	CDR3	FWR4	
H12V_L:	57	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC--	QQSYSTPI------	TFGQGTKLEIKR	108
H12V_H:	67	RVTITADEATSTAYMELNSLRSEDTAVYYCAR--	GGWCTGDCDAR--	TFVWFEPWGCGTTVTVSS	127

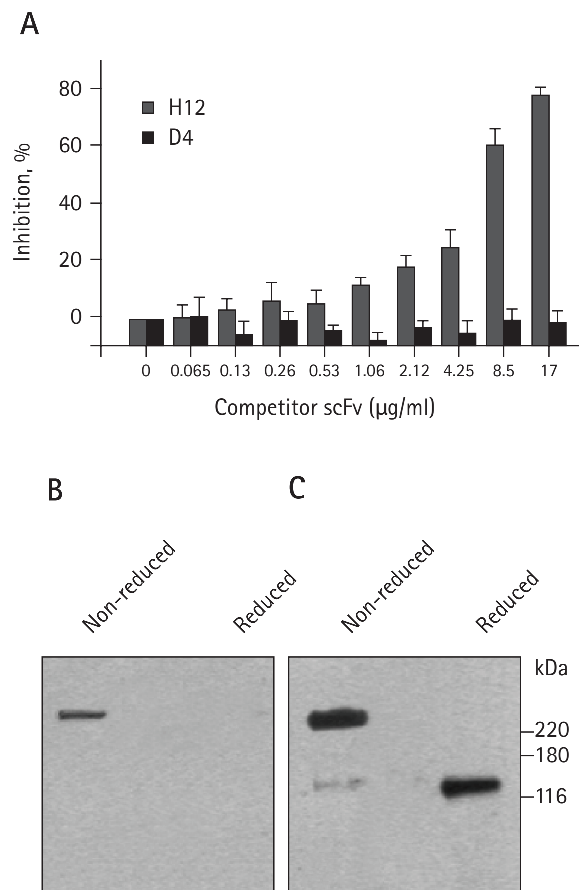
Framework regions 1–4 (FW1–4), and complementarity-determining regions 1–3 (CDR1–3) from both the V_H and V_L were determined by using the Vbase sequence directory. Based on its sequence, the scFv H12 is identified as V_{H1} family and $V_{\kappa 1}$ family.

Figure 3. Expression, purification and detection of scFv H12



(A) SDS-PAGE and **(B)** Western blot analysis the scFv H12 before (lane 1) and after (lane 2) purification. The scFv H12 was purified and refolded at the same time by gel filtration through a column with sephacryl S200HR. The membrane with scFv H12 was blotted by using anti-His-6-Tag antibody.

Figure 4. The scFv H12 binds to a conformational epitope within oligomeric S1-Fc proteins



(A) Competition ELISA showing the ability of scFv H12 to abrogate the binding of SARS sera to SARS-CoV, whereas the D4 scFv failed to bind to SARS-CoV. **(B)** The scFv H12 specifically recognizes S1-Fc proteins in oligomeric form under non-reduced condition, but it does not bind to S1-Fc proteins in monomeric form under reduced condition. **(C)** Anti human-Fc antibody used as a control recognizes S1-Fc proteins under both conditions.

In comparison, the anti-human Fc antibody recognized S1-Fc protein under both conditions (Figure 4C). These results strongly suggest that the scFv H12 recognized a conformational epitope shared by oligomeric S1-Fc protein.

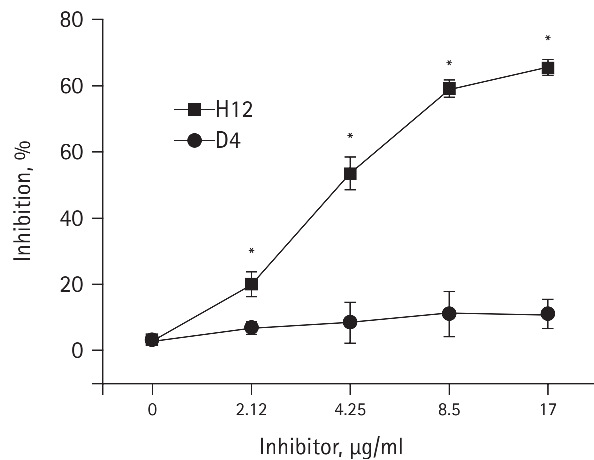
H12 neutralizing HIV/SARS S pseudovirus infection

HIV/SARS-S pseudovirus had been proved to be an efficient *in vitro* model for the study of SARS-CoV infection [32–34]. In this model, luciferase activity reflects the infection of pseudovirus in the host cells. Using HIV/SARS-S pseudovirus, we tested the neutralizing activity of scFv H12, and found that the scFv H12 significantly blocked the infection of Vero E6 cells by the SARS-S pseudovirus in a dose-dependent manner, whereas the control D4 antibody did not have any effect under the same conditions (Figure 5).

Table 2. Kinetic rates and binding affinity of scFv H12

*K*on, $M^{-1}S^{-1}$	*K*off, S^{-1}	K_a, M^{-1}	K_d, M
7.5×10^{3}	6.6×10^{-4}	1.36×10^{7}	7.35×10^{-8}

Figure 5. A HIV/SARS-S pseudovirus system is used as an infection model to evaluate the neutralizing activity of scFv H12



Luciferase activity, which reflects the infection and replication of pseudovirus, was measured by a luminometer. Compared with a control scFv D4, the scFv H12 significantly inhibited the infection of HIV/SARS S pseudovirus into Vero E6 cells and this inhibition was in a dose-dependent manner. Each point represents the mean of triplicate determinations; error bars ±SD. *Statistically significant differences ($P\leq0.01$).

Discussion

Antibodies have been used for a century for the prevention and treatment of infectious diseases. In viral disease, antibodies block viral entry into uninfected cells, promote antibody-directed, cell-mediated cytotoxicity by natural killer cells. It has been shown that S protein is, as a dimer or trimer, located on the surface of SARS virion, and binds to the receptor to initiate virus entry into host cells [9,11].

In order to obtain a neutralizing antibody that mimics natural antibody elicited by a viral pathogen, we constructed an immunized scFv library derived from B cells of four SARS patients with high titres of SARS virions. This B cell repertoire contained a variety of antibodies with high specificities and affinities for SARS-CoV. Compared with naive or synthetic antibody libraries, the immunized library is more likely to contain natural antibody with high specificity and affinity. Another important approach to increase the chances of obtaining natural antibody is using complete SARS-CoV virions, rather than a single recombinant protein, as an antigen for screening. The viral particle antigen bears natural and intact conformational immunogenic determinants. Previous studies by us and others have demonstrated that SARS virions bear trimeric S protein on their surface, and recombinant S protein tends to oligomerize to form a dimer or trimer [9–11,35]. The results from anti-HIV antibodies also showed that the HIV-neutralizing antibodies recognize a conformational epitope. Hadlock *et al.* studied the relationship between defined linear and conformational epitopes within HIV gp46 and found that the serum antibodies from infected individuals predominantly bind to a conformational epitope on native gp46 rather than denatured gp46 [36]. Quentin *et al.* also showed that virus neutralization correlated broadly with antibody binding to the oligomeric rather than the monomeric form of gp120 [37]. Thus, it was more feasible to select neutralizing antibody using whole SARS-CoV virions rather than using a single recombinant S protein.

Here we selected scFv H12 with high affinity for binding to SARS-CoV particles *in vitro*. Unlike other anti-monomer S1 antibodies [16–18] selected using a recombinant S1 protein from non-immunizing antibody libraries, the scFv H12 recognized oligomeric S1-Fc but did not bind to monomeric S1-Fc. It seems that the scFv H12 epitope consists of two S1-Fc subunits. Although we are not able to localize the scFv H12 epitope more precisely because scFv H12 does not recognize a linear epitope, it is clear that the scFv H12 epitope is different from that of antibodies derived from non-immunizing antibody libraries. These different binding patterns might be due to the use of different antigens and different libraries.

The competition assay showed that the scFv H12 was able to abrogate the binding of SARS immune sera to SARS-CoV, suggesting that scFv H12 bears a similar antigen epitope to native SARS antibodies in the sera. The neutralizing ability of scFv H12 may be via its blocking the conformational epitope on SARS virions.

In summary, we described an effective method to obtain an antibody that mimics the natural antibody in immune sera, by using SARS virions, rather than a single recombinant protein, as antigens for selection from an immunized antibody library. This approach may have significance not only for developing neutralizing antibodies, but also for better understanding the immunogenic characteristics of SARS protein and the design of SARS vaccine. In addition, this study highlights the usefulness of antigen-presenting systems that preserve epitope conformation in the investigation of immune responses. It will be interesting to study the conformationally sensitive epitope by extensive mutational analysis.

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